Two isoforms of *Saccharomyces cerevisiae* Glutaredoxin 2 are expressed *in vivo* and localize to different subcellular compartments

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SUMMARY

Glutaredoxin 2 (Grx2) from *Saccharomyces cerevisiae* is a member of the 2-Cys (dithiol) subfamily of glutaredoxins involved in the defense against oxidative stress in yeast. Recombinant yeast Grx2p expressed in *E. coli*. behaves as a "classical" glutaredoxin that efficiently catalyzes the reduction of hydroxyethyl disulfide by GSH. Grx2p also catalyzes the reduction of GSSG by dihydrolipoamide with even higher efficiency. Western blot analysis of *S. cerevisiae* crude extracts identifies two isoforms of Grx2p of 15.9 and 11.9 kDa, respectively. The levels of these two isoforms reach a peak during the exponential phase of growth in normal YPD medium, with the long form predominating over the short one. From immunochemical analysis of subcellular fractions it is shown that both isoforms are present in mitochondria but only the short one is detected in the cytosolic fraction. On the contrary, only the long form is prominent in microsomes. Mitochondrial isoforms should represent the processed and unprocessed products of a new ORF (YDR513W) with a putative start codon 99 bp upstream of the *GRX2* start codon so far described. These results indicate that GRX2 contains two in-frame start codons and that translation from the first AUG results in a product which is targeted to mitochondria. The cytosolic form would result either by initiation from the second AUG or by differential processing of one single translation product.

INTRODUCTION

Glutaredoxins (Grx) are small proteins (≈10 kDa), highly conserved throughout evolution, which catalyze GSH-disulfide oxidoreductions [1]. The first function assigned to glutaredoxin was as electron donor for the reduction of an intramolecular disulfide in ribonucleotide reductase [2] by means of a dithiol mechanism [3]. The active site sequence (Cys-Pro-Tyr-Cys) responsible for this activity is conserved in most species. However, only one of these cysteines is involved in other important reactions also catalyzed by Grx *in vitro*, such as the GSH-dependent reduction of protein-glutathionyl-mixed disulfides (protein-SSG), the so called deglutathionylase or dethiolase activity [4-6]. Since oxidative conditions induce the accumulation of protein-SSG [7, 8], Grx may play a role in the defense of cells against reactive oxygen species (ROS) by means of its dethiolase activity. Evidences for a protective role of Grx under oxidative conditions have been reported from biochemical [5, 6, 9, 10] and genetic [11, 12] approaches.

Two genes encoding glutaredoxins have been characterized in *Saccharomyces cerevisiae*, *GRX1* and *GRX2*, which share a high degree of similarity with other known Grxs from different species [12, 13]. Grx1p and Grx2p appear to play different roles in yeast, i.e., *GRX1* mutants are sensitive to oxidative stress caused by superoxide anion as induced by menadione whereas *GRX2* mutants are sensitive to stress induced by hydrogen peroxide; in addition, Grx2p accounts for most of the GSH-dependent oxidoreductase activity in yeast cells [12]. Moreover, *GRX1* and *GRX2* show different expression patterns under various stress conditions although both genes possess and are regulated by STRE elements [14].

A group of three additional genes named *GRX3*, *GRX4* and *GRX5* with homology to glutaredoxin have also been identified in yeast [11]. The proteins encoded by these genes show a marked difference with other glutaredoxins in that they only have one cysteine in the active site instead of two. This might be a substantial

property that would limit the action of this subfamily of glutaredoxins to processes that occur through a monothiol mechanism. Mutants lacking any of these three proteins showed a marked decrease (≈60%) in Grx activity, as determined by the standard assay in crude extracts, and were more prone to protein oxidative damage and more sensitive to osmotic stress than the wild type. In addition, *grx2 grx5* mutant was non-viable indicating that Grx2p and Grx5p can functionally substitute for each other; the triple mutation *grx3grx4grx5* was also lethal [11].

A new mammalian glutaredoxin has been recently characterized that specifically localizes in the mitochondria and the nucleus and is expressed in two isoforms, one of them with an N-terminal extension that functions as mitochondrial targeting signal [15, 16]. From an analysis of the genomic DNA sequence of *Saccharomyces cerevisiae* we have observed that the *grx2* gene coding for glutaredoxin-2 (thioltransferase) [13] has a potential in frame start codon 99 bp upstream of the so far described initial methionine, that would define a different ORF (YDR513W) (Fig 1). YDR513W would code for a protein longer than Grx2p with a N-terminal extension of 34 residues. To check whether this peptide constitutes a mitochondrial signal we have studied the subcellular localization of Grx2p.

We show here that Grx2p can be targeted to mitochondria or to the cytosol, and a mechanism that accounts for this differential localization is proposed and discussed.

EXPERIMENTAL

Materials.

All reagents employed were of analytical grade and were purchased from Sigma, unless otherwise specified.

Strains and growth conditions.

The yeast strains FY1679 (*MAT***a** *ura3-52 his3*Δ*200*) and CML235 (*MAT***a** *ura3-52 leu2*Δ*1 his3*Δ*200*) were used as wild types. *grx2* (MML44) and *grx5* (MML19) mutants were obtained from the wild type CML235 as described [11] and were kindly provided by Dr. E. Herrero (Universitat de Lleida, Spain). Yeast cells were grown at 30°C in YPD medium. *E. coli* BL21(DE3) was employed as a host for DNA cloning.

Cloning and expression of recombinant yeast Grx2p.

Two primers were designed (yGrx2-*Nde*I, 5'-CTACTCCACAT**ATG**GTATCCCAGGAAACAG-3', forward and yGrx2-*Bam*HI, 5'-TTAGCGGGATCCAAA**CTA**TTGAAATACC-3', reverse) to clone the *GRX2* gene according to the sequence obtained from the data base (*Saccharomyces* Genome Data Bank, locus GLRX). The forward primer contained the initiation codon (bold letters) and an *Nde*I site (underlined) and the reverse primer contained the stop codon (bold letters) and a *Bam*HI site (underlined). The primers were used to amplify *S. cerevisiae* genomic DNA by PCR (35 cycles at 96°C for 1 min, 50°C for 1 min and 68°C for 1.5 min) with Expand Long Template System (Roche Molecular Biochemicals). The PCR product was cloned into the pGEM-Te Vector System (Promega) and sequenced. The amplified fragment was subcloned into the pET-15b expression vector (Novagen) fused at the N-terminus to a 20 aminoacid peptide containing a polyhistidine tag and a thrombin cleavage site. The construct, named pET-15b-Grx2(s) was verified by PCR using the above primers and *E. coli* cells were transformed. Single colonies were selected and inoculated in LB medium containing 1

µg/ml ampicillin and were grown at 37°C until A_{600} = 0.5; the recombinant protein was induced with 0.5 mM IPTG and the cells were kept growing at room temperature overnight. Collection, lysis and crude extract preparation was performed as described [17]. His-tagGrx2p was purified from the extract by chromatography on a Talon Resin column (Clontech) and the His-tag was subsequently removed by controlled incubation with thrombin, as described before [18].

Assay of enzymatic activities.

Glutaredoxin activity was determined spectrophotometrically by measuring the reduction of 0.5 mM HED (Aldrich Chemicals) by 0.5 mM GSH in the presence of NADPH and 0.5 Units of yeast glutathione reductase (Sigma G-3664) at room temperature following the disappearance of NADPH at 340 nm [19]. One unit is defined as the oxidation of one µmol NADPH/min. Peroxidase activity was determined by substituting HED with peroxide substrates as indicated where appropriate.

Glutaredoxin dependent reduction of 0.5 mM GSSG by 0.64 mM dihydrolipoamide, was measured spectrophotometrically in the presence of 0.36 mM NADH and 1.7 Units of lipoamide dehydrogenase (Sigma L-2002), following the disappearance of NADH at 340 nm. One unit of dihydrolipoamide-GSSG reductase activity is defined as the oxidation of one µmol of NADH/min. Organelle marker enzymes were assayed according to published procedures: cytochrome *c* oxidase [20] was used as a mitochondrial marker, glucose-6-phosphate dehydrogenase [21] as a cytosolic marker and NADPH-cytochrome *c* reductase as a microsomal marker [22].

Preparation and purification of polyclonal antibodies to yeast Grx2p.

Recombinant Grx2p was used to immunize New Zealand albino rabbits according to Harboe and Ingild [23]; it was immobilized onto CNBr-activated Sepharose-4B (Amershan Pharmacia) and packed in a column. Immune sera were loaded and recycled several times; after washing with 2 M NaCl, elution was afforded with 0.5 M formic acid followed by rapid neutralization with 2 M Trizma**.**

Subcellular fractionation of yeast cells.

Cells of *S. cerevisiae* grown in YPD medium until stationary phase were harvested and subjected to subcellular fractionation as described [24]. Spheroplasts were prepared incubating with 0.5 mg/g cells lyticase in 20 mM potassium phosphate, pH 7.4 containing 1.2 M sorbitol. Spheroplasts were harvested by centrifugation, diluted in 20 mM MES-KOH pH 6.0, 0.6 M sorbitol, 0.5 mM PMSF (buffer A) and disrupted in a Dounce homogenizer. The crude mitochondrial fraction was separated by differential centrifugation with buffer A and finally collected at 12,000 x g and suspended in buffer A. The purity of this mitochondrial preparation was further increased by ultracentrifugation in 14% - 18% Nycodenz (Sigma). Centrifugation at 100,000 x g for 1.5 h provided the cytosolic fraction, which was dialyzed against 20 mM Tris-HCl pH 8.0, 0.1 M NaCl; and the microsomal fraction, which was suspended in 50 mM potassium phosphate pH 7.4, 0.1 M NaCl.

SDS-PAGE and Western blotting.

SDS-PAGE was performed on homogenous 15% acrylamide gels followed by Coomassie staining or electrophoretic semi-dry transfer to nitrocellulose membranes. The membranes were processed by the method of Towbin [25]. The primary antibodies were used at 1:500 dilution and developed with the chemiluminiscence ECL system (Amershan Pharmacia). The amount of Grx in the blots was calculated densitometrically (Syngene) using pure Grx as standard.

Protein concentration was determined spectrophotometrically [26] (Bio-Rad) using ovoalbumin as standard.

RESULTS

Sequence analysis of yeast Grx2.

Analysis of genomic DNA of *Saccharomyces cerevisiae* around *GRX2* gene shows an in frame start codon 99 nucleotides upstream of the canonical ATG (ORF YCL035C), that defines ORF YDR513W (Fig 1A). Translation of this new ORF would produce a 15.9 kDa protein similar to the so far described yeast Grx2 bearing a 34 residue N-terminal extension peptide. Prediction of subcellular localization using TragetP [27] identifies residues 1-34 as a signal peptide. This signal peptide bears a highly hydrophobic stretch between residues 9 and 26, is rich in basic residues, with a theoretical pI=10.0 (Fig 1), which are usual features of peptides bound for targeting and translocation to mitochondria [28]. Curiously enough, this presequence has acidic residues, but despite this unusual property the signal peptide meets most of the requirements to be a substrate of mitochondrial processing peptidase (MPP) [29] (Fig 1B). These include a set of three basic distal residues that will be located at the same side of an α-helix thus allowing interaction with the α subunit of MPP; a helix-linker-helix structure around the breaking peptide bond that would be between M35 and V36; a hydrophobic and a hydroxyl pair of residues at 1 and 2 positions relative to the cleavage site; and a proximal basic residue at -2 position relative to the cleavage site, arginine in the consensus pattern, but here substituted by lysine.

cDNA cloning, purification and characterization of recombinant Grx2p.

Grx2 expressed as His-tagged derivative was purified from bacteria by affinity chromatography on a Talon-metal affinity column. It was subsequently subjected to controlled digestion by thrombin to remove the His tag, as described in Experimental. The purity and size of the protein was verified by SDS-PAGE (Fig. 2A). Recombinant Grx2p was tested for its ability to catalyze thiol-disulfide oxidoreductions by means of the usual standard assay of Grx and was compared to human Grx1. Grx2p efficiently catalyzes the reduction of HED by GSH (59.4 ± 6.6

U/mg; kcat=713 min^{-1}) though with lower efficiency than that of human Grx1(180 ± 21.8 U/mg) and rat Grx1 (137.8 ± 39.6 U/mg) under equivalent conditions, but higher than that of human Grx2 (37 ± 1 U/mg [16]; 3 U/mg [15]). Reduction of GSSG by dihydrolipoamide, was catalyzed by yeast Grx2p with high efficiency (71.5 ± 23.9 U/mg; kcat=858 min^{-1}). A recombinant truncated form of Grx5p that lacks 18 residues at the N- terminus was also produced and proved completely unable to catalyze any of the reactions tested above (data not shown). Since Grx5 and Grx2 have been ascribed protective antioxidant roles [11] and it has been shown that Grx acts as electron donor for human plasma GSH-Peroxidase [30], we also tested for the reduction of three peroxides, cumene-, *tert*-butyl- and hydrogen peroxides. None of the proteins showed any trace of peroxidase activity.

Recombinant Grx2p was used to obtain polyclonal antibodies from rabbits and to purify them by immunoaffinity chromatography. The affinity purified anti-Grx2p preparation was used to study the expression of the protein in cells of *Saccharomyces cerevisiae* by Western blotting (Fig. 2B). Anti-Grx2 antibodies recognize two bands in Western blots of *S. cerevisiae* cells: one larger (15.9 kD) than recombinant Grx2p and one that coincides (11.9 kD). The proteins are expressed in wild type and *grx5* strains in the stationary phase; as expected, the *grx2* mutant is negative with anti-Grx2 demonstrating that both bands are products of *GRX2*. Therefore yeast Grx1p, which is present in all three strains, does not cross-react with the antibodies. The same lack of cross-reactivity is also true for Grx5p and human Grx1 (Fig. 2B).

We have also followed the levels of both isoforms along the normal growth in YPD medium and have found that both proteins reach a peak during the exponential phase but their relative proportions vary, the long form being predominant at the exponential phase (see Fig 3).

Subcellular localization of Grx2.

Cells of *S. cerevisiae* grown to stationary phase were collected and subjected to subcellular fractionation as described in Experimental. Preparations of cytosolic and mitochondrial fractions were obtained by differential centrifugation and

crossed contamination between them was unequivocally discarded as judged by their levels of marker enzymes (Table 1). As expected, a subfraction of the endoplasmic reticulum, the so called mitochondria-associated membrane (MAM) [31], co-isolates with mitochondria. The microsomal fraction showed “membrane leakage” from other subcellular fractions.

A Western blot analysis was performed with affinity purified anti-Grx2 antibodies and from the results shown in Fig. 4. we conclude that both isoforms of Grx2p localize in mitochondria. The above mentioned two bands observed in crude extracts of S. *cerevisiae* are present in the mitochondrial fraction, whereas only the short form is detected in the cytosolic fraction and only the long isoform is predominantly detected in microsomes.

DISCUSSION

Grx2p, the product of ORF YCL035C from *Saccharomyces cerevisiae*, has been cloned and its subcellular distribution has been studied by Western blotting. In doing so, we have detected two isoforms of different sizes, expressed at the same time in yeast cells.

When compared to other Grx2 from human, rat and mouse, yeast Grx2p shows 29% -30.5% similarity. The mature protein devoid of presequence has a pI=5.76, more acidic than the majority of mammalian Grxs but conserves all the structural features that define a 2-Cys (dithiol) subfamily of glutaredoxins [32] including the catalytic site, the glutathione binding site and structurally important hydrophobic domains (Fig. 5). However, at difference with other Grx2, yeast Grx2p has conserved proline at the active site instead of serine [15, 16].

Recombinant Grx2p also displays all the catalytic properties of glutaredoxins [33]. Its catalytic efficiency in the standard assay is lower than that of human and rat Grx1, despite its 100% identity at the active site motif CPYC, but higher than that of human Grx2 in which active site Pro is substituted by Ser. Lack of glutathione peroxidase activity is a striking property of Grx2p in the light of a recent report [34] in which Grx1p catalyzes peroxide reduction by GSH. These data further support the proposed functional differences between both yeast dithiol glutaredoxins [14].

On the other hand, yeast Grx2 is very efficient as a dihydrolipoamide GSSG oxidoreductase, what may be of interest for a mitochondrial enzyme. Actually, the ratio of this activity to the standard GSH-HED oxidoreductase activity is higher in the mitochondrial than in the cytosolic fraction. Lipoic acid is a redox coenzyme of several enzymes and multienzyme complexes localized in mitochondria. A link between lipoic and glutathione pools in the mitochondria would have interesting consequences in the context of antioxidant defense and Grx could afford the connective role.

The 11.9 kDa isoform detected in the cytosolic fraction would represent the putative product of YCL035C, (Fig. 1) i.e., the “classical” yeast glutaredoxin (thioltransferase) initially described and studied by Gan and coworkers [13] with

resemblance to Grx1 from other organisms, which lack any organelle targeting presequence. However, translation from the second AUG has to be confirmed by further experiments.

The hydrophobicity of the presequence may be responsible for membrane leakage and the presence of the long isoform in the microsomal fraction. It would be of interest to ascertain whether the membrane associated form is actually an immature species trapped in the mitochondrial import machinery or it plays specific roles in membranes.

Nuclear-encoded mitochondrial proteins are synthesized as larger precursor molecules that, upon import into mitochondria, are subject to proteolytic cleavage, The two Grx2p isoforms present in mitochondria should correspond to the processed and unprocessed products of a transcript encoding a N-terminal mitochondrial target peptide. ORF YDR513W fulfills this requirement as described above (see Fig. 1). The size of the long isoform detected in crude extracts and in mitochondrial and microsomal subcellular fractions agrees with the size of a putative peptide resulting from translation of YDR513W.

The presence of two forms of Grx2 in the mitochondrial fraction would be due to differences in the rates of membrane translocation and proteolytic processing of one single translation product by matrix peptidases. The rationale for this explanation is based on similarities with yeast fumarase, for which a fraction of the protein folds outside mitochondria into an import incompetent state that upon cleavage is released by retrograde movement into the cytosol [28]. The rate of proteolytic cleavage could be intrinsically slow for Grx2p due to the presence of Lysine at -2 position relative to the cleavage site, instead of Arginine, since it is known that substitution of this Arginine in model peptides greatly decreases the efficiency of the protease [29]. Slow processing would then allow for Grx2p to fold before import and would thus return to the cytosol. Only the fraction of Grx2p that is cleaved before folding would be imported into the matrix. This is our working hypothesis for further research.

Alternative splicing is the mechanism that operates in mammals to target Grx2 either to the mitochondria or to the nucleus [15, 16]. In *Saccharomyces cerevisiae*,

whose coding DNA is not interrupted by introns, more rudimentary solutions seem to operate that consist of either overlapping ORFs and alternative AUG selection [35, 36] or slow processing and retrograde movement [37, 38], as might be the case for Grx2 as discussed above. An intermediate situation may function in the fission yeast *Schizosaccharomyces pombe*, whose glutaredoxin gene contains three introns [39] and also bears an "additional" in-frame start codon 90 bp upstream of the "main" start codon (GeneBank *S. pombe* genomic DNA). Furthermore, the use of alternative translation initiation sites combined with alternative splicing may increase the complexity of the population of Grx isoforms as has been suggested recently [15].

Whether translation occurs from both start codons or there exists one single translation product, and what possible regulatory mechanisms might be involved to control the proportion of cytosolic and mitochondrial isoforms, are interesting questions for further investigations, that will shed more light on novel ways to control postgenomic events in eukariotic cells.

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ABBREVIATIONS

DTT, dithiothreitol; Grx, Glutaredoxin; MPP, mitochondrial processing peptidase; ORF, open reading frame; protein-SSG, protein-glutathionyl mixed disulfide; ROS, reactive oxygen species; STRE, stress response element; YPD, yeast extract peptone dextrose medium.

LEGENDS TO THE FIGURES

Figure 1: Sequence analysis of Grx2p from *Saccharomyces cerevisiae*. A). The upper line shows a stretch of genomic DNA sequence of *S. cerevisiae* comprising two start codons that define open reading frames YDR513W and YCL035C (as indicated. The N-terminal putative presequence is shadowed and the calculated molecular weight of the protein is indicated. **B)** Analysis of the N-terminal extension.. Structural requirements that define this sequence as a precursor recognized and cleaved by matrix processing peptidase are indicated according to Ito (1999) [29] Number and position of distal basic residues vary among known extension peptides, whereas the most frequent proximal basic residue is arginine. A helix-linker-helix structure is needed for effective cleavage of precursors. Predicted secondary structure is also indicated underneath.

Figure 2. SDS-PAGE of purified recombinant Grx2p and Western detection in crude extracts. **A)** After IPTG induction, the protein was purified as the polyHis-Grx2 and was digested with thrombin under controlled conditions to eliminate the PolyHis tag. Lane 1, Bio-Rad pre-stained molecular weight markers; lane 2, Grx2p. Protein was stained with Coomassie Blue. **B)** Samples from cells of *S. cerevisiae* grown in YPD medium to stationary phase were collected by centrifugation, suspended in electrophoretic sample buffer and subjected to Western blot analysis with anti-Grx2 antibodies as described in Experimental. Lanes 1 and 8, unstained Mr markers; lane 2, 0.1 µg of yeast Grx2p; lane 3, 0.15 µg of yeast Grx5p; lanes 4 – 6, 20 µl of crude extracts from *wt, grx2* and *grx5* mutant strains, respectively; lane 7, 0,1 µg of human recombinant Grx1.

Figure 3. Expression of Grx2p along the growth of *Saccharomyces cerevisiae* in YPD medium.. **A)** 1 ml aliquots were withdrawn from the growth medium at intervals along the growth curve and were analyzed by Western blotting as indicated in Experimental: lanes 1 and 7, 0.1 µg of Grx2p; lanes 2 – 6, samples of 20 µl

taken at equivalent points from early exponential to late stationary growth phases for all three strains. **B)** Growth curve of *wild type S. cerevisiae* and concentration of long and short isoforms of Grx2p determined by quantitative densitometry from the blots expressed as ng/A_{600nm}. ◆--◆, absorbance at 600 nm; ▲--▲, Grx2p long isoform; ●--●, Grx2p short isoform; ■--■, total Grx2p.

Figure 4. Localization of Grx2p in subcellular fractions of *Saccharomyces cerevisiae*. Mitochondrial (mit), cytosolic (cyt) and microsomal (micr) fractions were obtained from cells of *S. cerevisiae* grown to stationary phase as described in Experimental. 25µg protein from each fraction was loaded onto SDS-PAGE and analyzed by Western blotting. 50 ng of Grx2p were included as a reference; MW, molecular weight standards.

Figure 5. Aminoacid sequence alignment of yeast Grx2 with human, rat and mouse Grx2. Mitochondrial and nuclear human Grx2 are shown [Lundberg, 2001 #206]. Residues conserved in at least three of the aligned sequences are boxed. S1 indicates the active site and S2 the glutathione binding site. Translation initiation sites of ORFs *YDR513W* and *YCL035C* are indicated by arrows.

Table 1. Enzymatic activities of subcellular fractions of *Saccharomyces cerevisiae*. The activities were measured by the methods described in the Experimental section. ([1]) Standard assay for Grx, that is the rate of HED reduction by GSH. ([2]) Rate of GSSG reduction by dihydrolipoamide. ([3]) The ratio of both activities. The data presented are the mean values ± s.d. of independent experiments, $n = 3$.

Subcellular fraction	Specific activities (mU/mg)					
	NADPH cyt c R'ase	G6PDH	Cyt *c* oxidase	Grx		
				HED[1]	Lipoamide[2]	Lip/HED[3]
Cytosol	5 ± 4	68 ± 1	0	98 ± 4	32 ± 4	0.3
Mitochondria	28 ± 14	0	46 ± 13	28 ± 5	28 ± 0.1	1
Microsomes	18 ± 8	10 ± 3	13 ± 4	5 ± 0.2	67 ± 3	13.4

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LEGENDS TO THE FIGURES

Figure 1: Sequence analysis of Grx2p from *Saccharomyces cerevisiae*. A). The upper line shows a stretch of genomic DNA sequence of *S. cerevisiae* comprising two start codons that define open reading frames YDR513W and YCL035C (as indicated. The N-terminal putative presequence is shadowed and the calculated molecular weight of the protein is indicated. **B)** Analysis of the N-terminal extension.. Structural requirements that define this sequence as a precursor recognized and cleaved by matrix processing peptidase are indicated according to Ito (1999) [29] Number and position of distal basic residues vary among known extension peptides, whereas the most frequent proximal basic residue is arginine. A helix-linker-helix structure is needed for effective cleavage of precursors. Predicted secondary structure is also indicated underneath.

Figure 2. SDS-PAGE of purified recombinant Grx2p and Western detection in crude extracts. **A)** After IPTG induction, the protein was purified as the polyHis-Grx2 and was digested with thrombin under controlled conditions to eliminate the PolyHis tag. Lane 1, Bio-Rad pre-stained molecular weight markers; lane 2, Grx2p. Protein was stained with Coomassie Blue. **B)** Samples from cells of *S. cerevisiae* grown in YPD medium to stationary phase were collected by centrifugation, suspended in electrophoretic sample buffer and subjected to Western blot analysis with anti-Grx2 antibodies as described in Experimental. Lanes 1 and 8, unstained Mr markers; lane 2, 0.1 µg of yeast Grx2p; lane 3, 0.15 µg of yeast Grx5p; lanes 4 – 6, 20 µl of crude extracts from *wt, grx2* and *grx5* mutant strains, respectively; lane 7, 0,1 µg of human recombinant Grx1.

Figure 3. Expression of Grx2p along the growth of *Saccharomyces cerevisiae* in YPD medium.. **A)** 1 ml aliquots were withdrawn from the growth medium at intervals along the growth curve and were analyzed by Western blotting as indicated in Experimental: lanes 1 and 7, 0.1 µg of Grx2p; lanes 2 – 6, samples of 20 µl taken at equivalent points from early exponential to late stationary growth phases for

all three strains. **B)** Growth curve of *wild type S. cerevisiae* and concentration of long and short isoforms of Grx2p determined by quantitative densitometry from the blots expressed as ng/A_{600nm}. ◆--◆, absorbance at 600 nm; ▲--▲, Grx2p long isoform; ●--●, Grx2p short isoform; ■--■, total Grx2p.

Figure 4. Localization of Grx2p in subcellular fractions of *Saccharomyces cerevisiae*. Mitochondrial (mit), cytosolic (cyt) and microsomal (micr) fractions were obtained from cells of *S. cerevisiae* grown to stationary phase as described in Experimental. 25µg protein from each fraction was loaded onto SDS-PAGE and analyzed by Western blotting. 50 ng of Grx2p were included as a reference; MW, molecular weight standards.

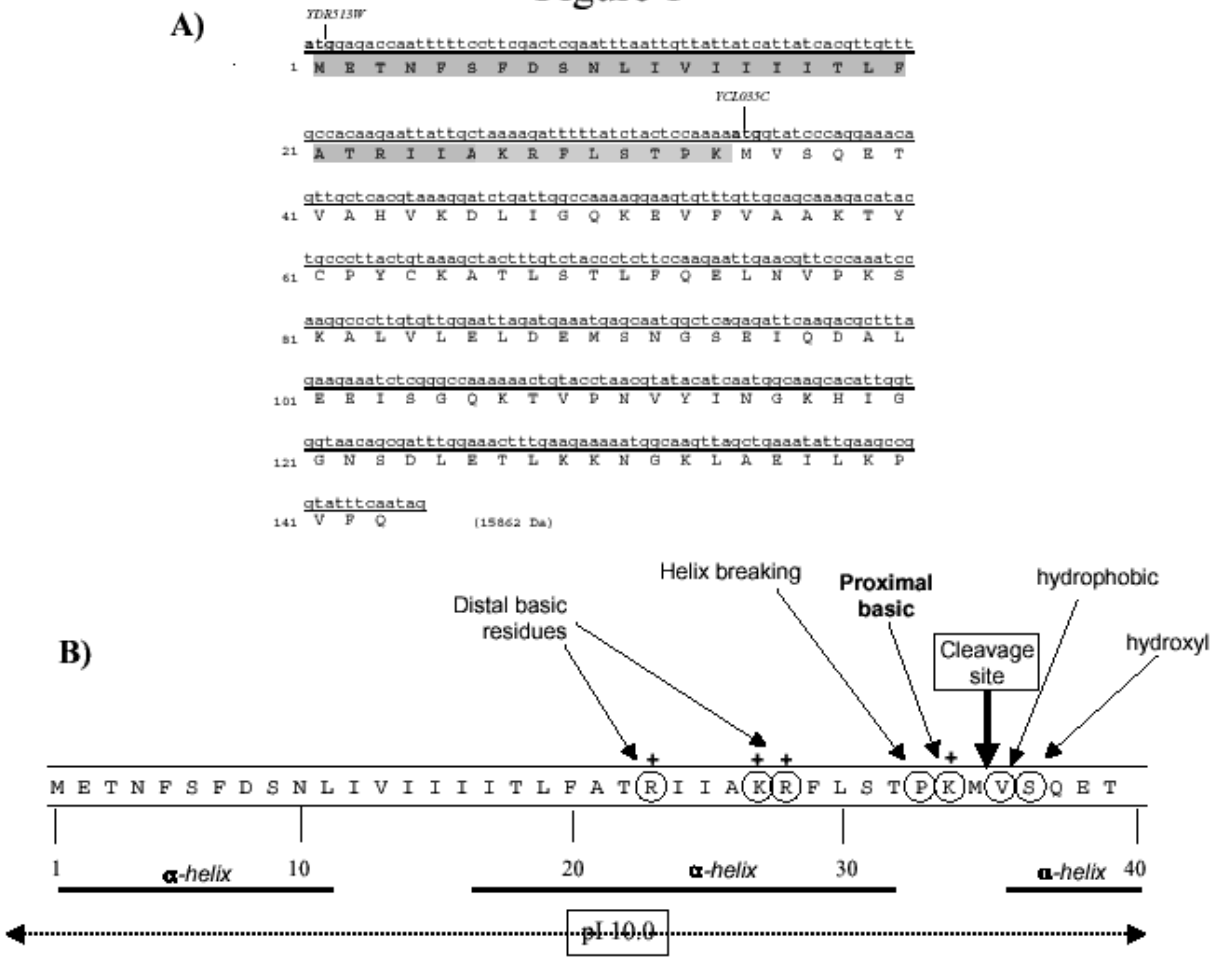
Figure 5. Aminoacid sequence alignment of yeast Grx2 with human, rat and mouse Grx2. Mitochondrial and nuclear human Grx2 are shown [Lundberg, 2001 #206]. Residues conserved in at least three of the aligned sequences are boxed. S1 indicates the active site and S2 the glutathione binding site. Translation initiation sites of ORFs *YDR513W* and *YCL035C* are indicated by arrows.

Table 1. Enzymatic activities of subcellular fractions of *Saccharomyces cerevisiae*. The activities were measured by the methods described in the Experimental section. ([1]) Standard assay for Grx, that is the rate of HED reduction by GSH. ([2]) Rate of GSSG reduction by dihydrolipoamide. ([3]) The ratio of both activities. The data presented are the mean values ± s.d. of independent experiments, $n = 3$.

Subcellular fraction	Specific activities (mU/mg)					
	NADPH cyt c R'ase	G6PDH	Cyt *c* oxidase	Grx		
				HED[1]	Lipoamide[2]	Lip/HED[3]
Cytosol	5 ± 4	68 ± 1	0	98 ± 4	32 ± 4	0.3
Mitochondria	28 ± 14	0	46 ± 13	28 ± 5	28 ± 0.1	1
Microsomes	18 ± 8	10 ± 3	13 ± 4	5 ± 0.2	67 ± 3	13.4

Pedrajas et al., Fig 1

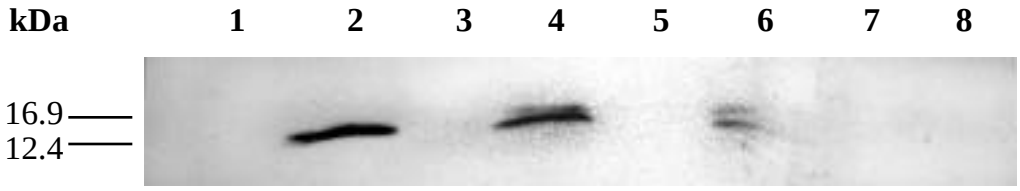
Figure 1



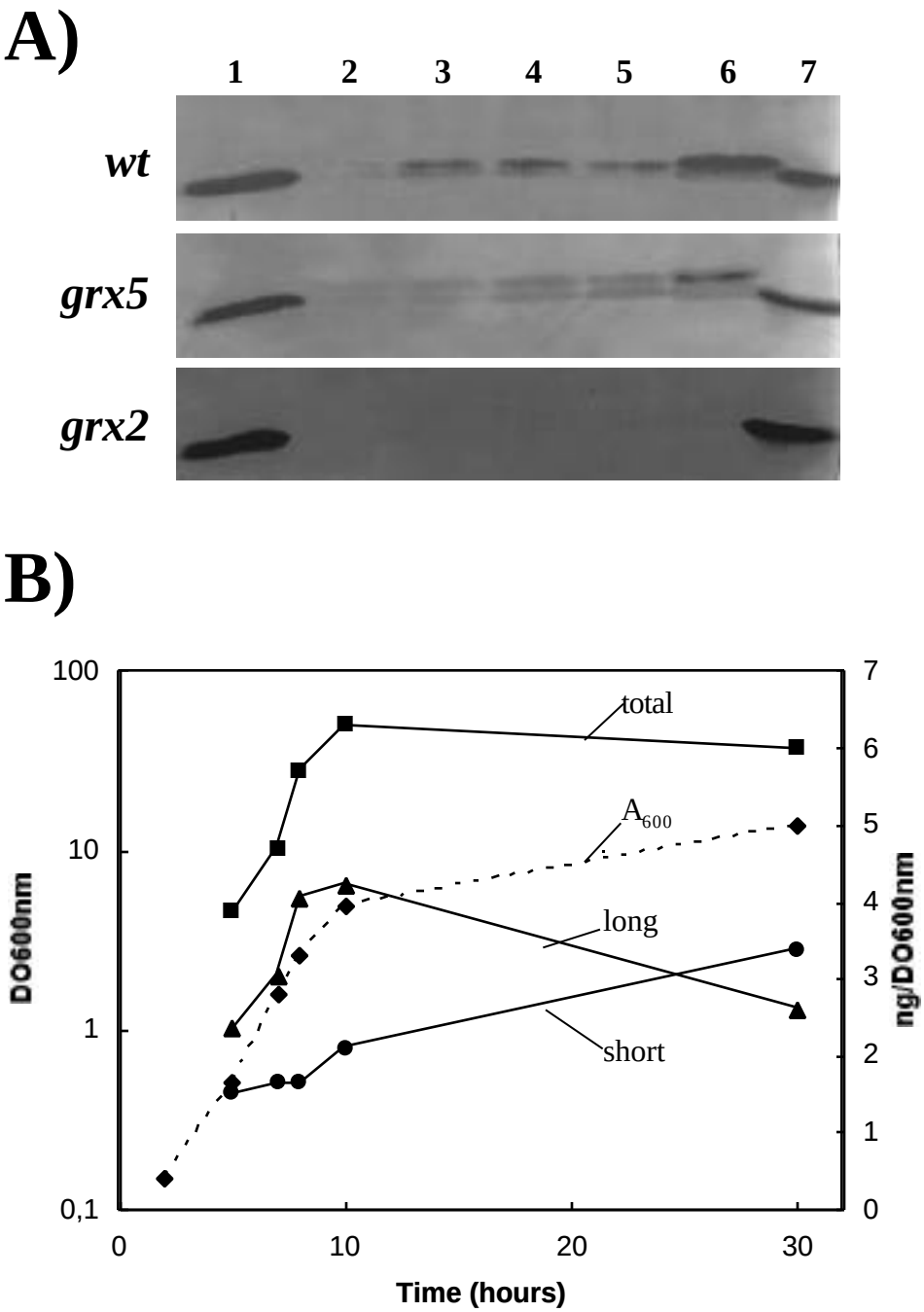
Pedrajas et al., Fig 2

A)

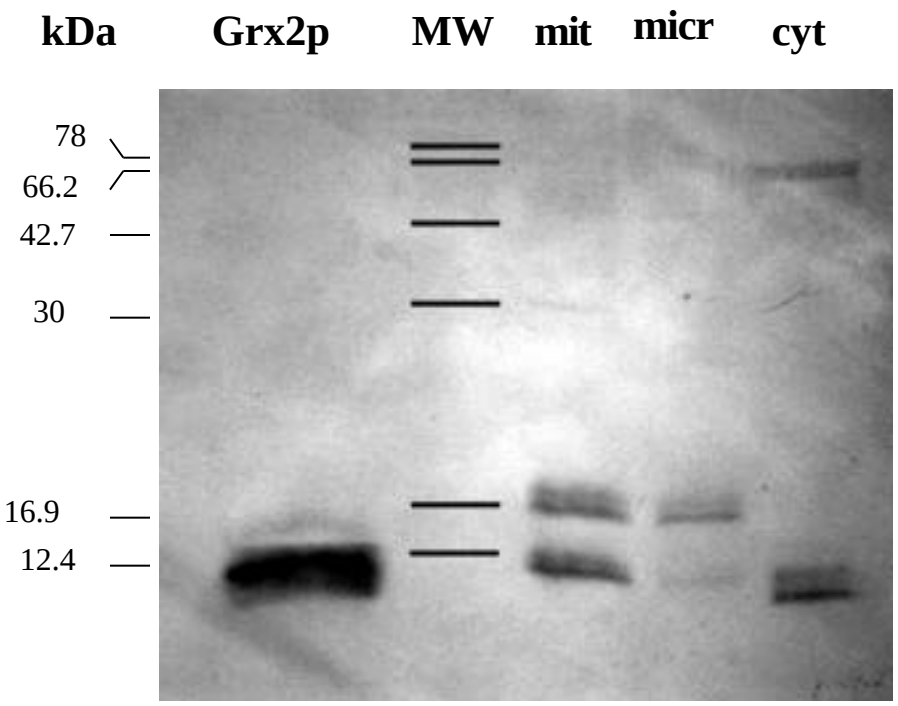
B)



Pedrajas et al., Fig 3



Pedrajas et al., Fig 4



Pedrajas et al., Fig 5